STIC-ILL

QD 501.A6

From: Baker, Maurie
Sent: Friday, August 01, 2003 9:56 PM
To: STIC-ILL
Subject: Please provide references ASAP

Please provide the following references ...ASAP
My mailbox is located in *** 3B01 ***
Thank you!

(1) Hoffman et al. 1992, Anal. Biochem. Vol. 203, pp. 70-75

(2) Kienhaus et al. 1992, J. Receptor Res. Vol. 12, pp. 389-399

For 09/248158

Maurie Garcia Baker, Ph.D.
Primary Examiner, Art Unit 1639
Crystal Mall 1, Rm. 3D11 (703) 308-0065

ANALYTICAL BIOCHEMISTRY 203, 70–75 (1992)

Characterization of a Scintillation Proximity Assay to Detect Modulators of Transforming Growth Factor α (TGFα) Binding

Richard Hoffman[1] and Lewis Cameron
Xenova Ltd., 545 Ipswich Road, Slough, SL1 4EQ, England

Received September 23, 1991

A scintillation proximity assay (SPA) for transforming growth factor α (TGFα) using SPA beads coated with A431 membranes has been studied. Binding of TGFα to the beads was characteristic of a receptor interaction. A class of high-affinity receptors for [^{125}I]-TGFα (K_d = 0.10–0.26 nM) was detected by competition studies between [^{125}I]TGFα and cold TGFα and by analysis of association and dissociation rate constants. An antibody to the epidermal growth factor receptor (clone 528) inhibited binding of [^{125}I]TGFα (IC50 = 0.20 μg/ml), but an anti-TGFα antibody (clone 134A–2B3) (<25 μg/ml) did not block binding. Suramin inhibited [^{125}I]-TGFα binding (IC50 = 0.20 mM). The ether lipids 1-*O*-hexadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine, and *rac*-lyso-platelet activating factor inhibited TGFα binding (IC50 values of 49, 69, and 57 μM, respectively). SPA is a convenient method for identifying agents that may act by interfering with TGFα binding.

The epidermal growth factor (EGF) receptor is frequently overexpressed in human carcinomas of the esophagus (1), head and neck (2), bladder (3), and breast (4). In the latter two cases, overexpression of the EGF receptor is associated with poor prognosis. A family of structurally related ligands bind to the EGF receptor. These include EGF, TGFα, amphiregulin, and the pox virus growth factors (5). TGFα has been detected in effusions from a variety of tumors (6), and is coexpressed with the EGF receptor in some tumors and transformed cell lines (7–9). These observations support studies with anti-TGFα antibodies (10,11) that indicate that TGFα can stimulate growth via the EGF receptor in an autocrine loop. Recent studies with transgenic mice have confirmed the importance of TGFα in cellular proliferation and neoplastic transformation (12–14). In contrast, there is no strong evidence for a role for EGF in tumorigenesis.

Attempts to identify ligand antagonists of the EGF receptor have so far met with limited success. Structure–activity studies with fragments of TGFα indicate that the receptor binding domain of TGFα is formed by multiple separate regions of the peptide (15). A modified peptide fragment of EGF blocks binding of EGF to the EGF receptor, but it probably acts indirectly through an alternative binding site (16). The nonpeptide antagonist suramin has been reported to antagonize EGF binding, but this substance is not specific for EGF (17).

In order to screen for ligand antagonists of the EGF receptor, a rapid, robust, reproducible, and physiologically relevant assay is required. Several assays presently used are based on binding of [^{125}I]EGF to the EGF receptor present on membranes or on immobilized cells of the cell line A431 (18). Biotinylated EGF has been used in place of radiolabeled EGF (19). Recently, scintillation proximity assays (SPA) have been described (20). These assays are based on fluor-containing beads coated with an appropriate receptor. Only bound ligand excites the fluor and consequently bound and free ligand do not need to be separated prior to quantitation. We have evaluated a SPA system based on beads coated with membranes from A431 cells. We chose [^{125}I]TGFα as the competing ligand since TGFα, rather than EGF, is implicated in tumorigenesis, and the two ligands may bind differently to the EGF receptor (21).

[1] To whom correspondence should be addressed at present address: MRC Unit of Clinical Oncology and Radiotherapeutics, Hills Road, Cambridge CB2 2QH, England. Fax: (0223) 213556.

[2] Abbreviations used: EGF, epidermal growth factor; TGFα, transforming growth factor α; SPA, scintillation proximity assay; NSB, nonspecific binding.

0003-2697/92 $5.00

MATERIALS

[^{125}I]TGFα was purchased from Amersham International plc (Amersham, Bucks, England). Recombinant human TGFα was purchased from Bachem (UK) Ltd. (Saffron Walden, Essex, England) and receptor-grade murine EGF was from Sigma (Poole, Dorset, England). Murine monoclonal antibody clone 528 to the EGF receptor and murine monoclonal antibody clone 134A-2B3 to TGFα were purchased from Cambridge Bioscience (Cambridge, England). Murine monoclonal antibody clone 29.1 to the EGF receptor was from Sigma and murine monoclonal antibody clone EGFR1 to the EGF receptor was from Amersham International plc. All other chemicals were purchased from Sigma. Tissue culture media were purchased from GIBCO (Paisley, Scotland). SPA beads coated with membranes from A431 cells were purchased from Amersham International plc.

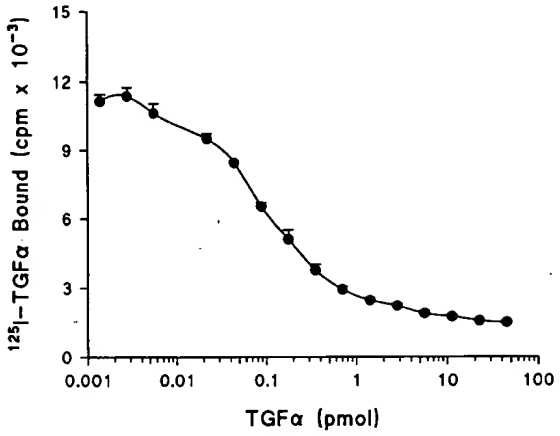

FIG. 1. Competition for [^{125}I]TGFα binding by unlabeled TGFα. Membrane/bead complex (100 μl) and [^{125}I]TGFα (76,760 dpm, 886 Ci/mmol; equivalent to 0.039 pmol) were incubated with various amounts of unlabeled TGFα (50 μl) for 2 h. Values represent means ± SE (n = 3).

METHODS

Scintillation Proximity Assay

The SPA beads contain a scintillant based on yttrium silicate and are coated with the polycationic polymer, polylysine. The A431 membranes (prepared from a postnuclear supernatant) carry a net negative charge and therefore bind to the polylysine. The binding of [^{125}I]TGFα to the A431 membranes brings the isotope in close proximity to the scintillant, allowing Auger electrons to stimulate the scintillant. Unbound ligand is not in close enough proximity to allow such energy transfer and hence no signal is generated.

[^{125}I]TGFα was diluted in buffer A (20 mM Hepes/0.1% bovine serum albumin, pH 7.5) to about 50,000 cpm per 50-μl aliquot. Precise activity was determined by counting a 10-μl aliquot in a gamma counter (Cobra) (counting efficiency, 75%) and adjusting the activity of the [^{125}I]TGFα if the counts varied by more than 20% from 50,000 cpm per 50-μl aliquot. Sample or cold EGF in buffer A (50 μl) was added to T-trays (Pharmacia) followed by [^{125}I]TGFα (50 μl). Bead/membrane complex, provided as a lyophilized preparation, was reconstituted by adding 40 ml buffer A. The reconstituted mixture was vigorously agitated and a 100-μl aliquot was added to the reaction mix. The T-trays were covered and shaken (250 rpm) for 2 h at room temperature. The samples were counted for 1 min in a beta plate reader (Model 1205, LKB). Bound ligand was estimated based on a counting efficiency of 50% for the beta plate reader and a counting efficiency of 60% for the bead/ligand complex (Bosworth, personal communication). Interbatch variation in the counting efficiency of the beads is ±10% (Bosworth, personal communication). Free ligand was determined by subtracting bound ligand from total ligand added. Nonspecific binding (NSB) was determined in the presence of 82 nM cold EGF. This concentration of EGF reduced [^{125}I]TGFα binding to levels equivalent to those acquired using an excess (100 nM) cold TGFα and was used in preference to TGFα in routine determinations of NSB values for reasons of cost. Unless otherwise stated, all experiments were carried out three times with triplicate determinations in each experiment. Results from typical experiments are presented.

Kinetic Analysis

Scatchard analysis was performed using LIGAND (22) as modified for microcomputer by McPherson (23). Association and dissociation rate constants were determined by the computer programme KINETIC (23). LIGAND and KINETIC are part of the McPherson computer package available from BIOSOFT (P.O. Box 10938, Ferguson, MO 63135). The goodness of fit of models (F value) was assessed on the basis of the "extra sum of squares" principle as described by Munson and Rodbard (22).

RESULTS

Binding of TGFα to the membrane/bead complex was investigated by incubating a fixed amount of [^{125}I]TGFα with increasing amounts of unlabeled TGFα. Unlabeled TGFα competed with [^{125}I]TGFα for binding in a dose-dependent manner. The concentration of unlabeled TGFα giving 50% inhibition in binding of [^{125}I]TGFα (IC50) was 0.34 ± 0.09 nM (average ± SE, n = 4). Results from a competition experiment following a 2-h incubation at room temperature are shown in Fig. 1. The shape of the competition curve remained essentially identical

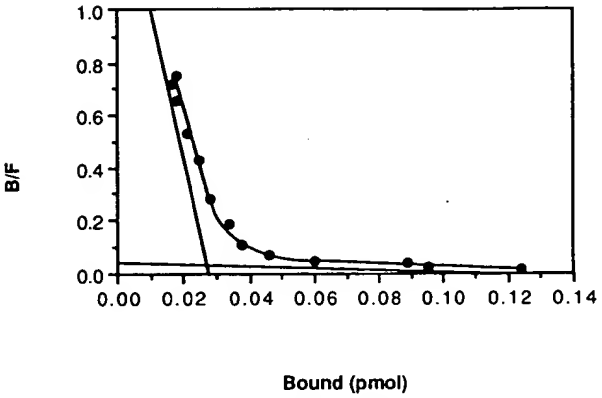

FIG. 2. Scatchard analysis of TGFα binding. The graph represents LIGAND analysis of the competition curve shown in Fig. 1.

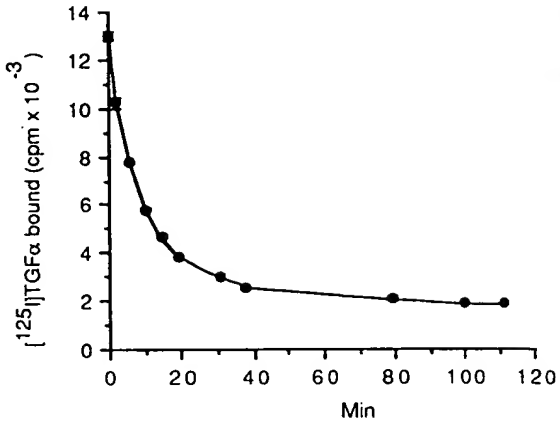

FIG. 3. Dissociation of [^{125}I]TGFα from the membrane/bead complex. Membrane/bead complex and [^{125}I]TGFα were equilibrated for 2 h; 82 nM unlabeled EGF was then added and the reaction mixes were assayed at intervals. The curve represents the fit determined by KINETIC assuming a biexponential model. Values represent means ± SE (n = 3).

over the next 20 h (data not shown). Subsequent experiments were incubated for 2 h.

When the data in Fig. 1 were transformed by Scatchard analysis a curvilinear plot was obtained (Fig. 2). These data were analyzed by LIGAND using a one-site model allowing the background value to float and by a two-site model. Analysis using a one-site model indicated an equilibrium dissociation constant (K_d) = 0.67 nM and maximum binding sites (B_{max}) = 429 pM. Analysis using a two-site model gave K_d values of 0.10 and 13.5 nM and B_{max} values of 151 and 386 pM, respectively. The F ratio test in the LIGAND program for comparing the best statistical fit between the two-site and one-site models gave an F value of 2.28 (P = 0.148), indicating that there is no evidence for a significant difference between the two models. Analysis of a further three experiments indicated a single class of receptors in two of the experiments and no evidence for a significant difference between the two models in a third experiment (F = 3.05; P = 0.104). Overall, these data probably indicate that the second class of sites is a binding artifact. A summary of the LIGAND analysis of several competition assays is shown in Table 1.

TABLE 1

Scatchard Analysis of TGFα Competition Curves

	K_d (nM)		B_{max} (pM)	
Experiment	Site 1	Site 2	Site 1	Site 2
1	0.25	250	80	1000
2	0.18	NI[a]	67	NI
3	0.26	NI	93	NI
4	0.10	13.5	151	386

Note. Membrane/bead complex and [^{125}I]TGFα were incubated with various amounts of unlabelled TGFα for 2 h as described under Methods.

[a] NI, no second site was identified by LIGAND.

The specificity of the interaction between [^{125}I]TGFα and the beads was next examined. The following peptide ligands caused no detectable reduction in binding of [^{125}I]TGFα to the beads: interleukin-1 (25 nM), tumour necrosis factor (25 nM), gastrin-releasing peptide (25 nM), basic fibroblast growth factor (25 nM), insulin-like growth factor (25 nM), transforming growth factor β (12.5 nM), and platelet-derived growth factor (5 nM).

The dissociation rate constant (k_2) of [^{125}I]TGFα from its receptor was determined by diluting equilibrated samples with a large excess of cold EGF (82 nM) and then quantifying the amount of bound [^{125}I]TGFα at intervals. The [^{125}I]TGFα dissociated from the bead/membrane complex in a time-dependent manner until a level similar to the level of NSB (about 2000 cpm) resulted (Fig. 3). When these data were transformed by KINETIC (23), a dissociation constant (k_2) = 0.037 min^{-1} was calculated assuming a monoexponential model and dissociation constants of 0.024 and 0.122 min^{-1} were calculated assuming a biexponential model. The statistically best fit was produced assuming a biexponential model (F = 112; P < 0.05).

The rate of binding of [^{125}I]TGFα to the beads was determined by measuring total and nonspecific binding every few minutes after the reaction was initiated. Triplicate samples were counted simultaneously for 1 min. As shown in Fig. 4, specific binding (total minus nonspecific) was maximal after approximately 120 min. Using KINETIC (23), an observed association constant (k_{obs}) = 0.092 min^{-1} was calculated assuming a monoexponential model and association constants of 0.032 and 1.07 min^{-1} were calculated assuming a biexponential model. The data were best fitted to a biexponential model (Fig. 5).

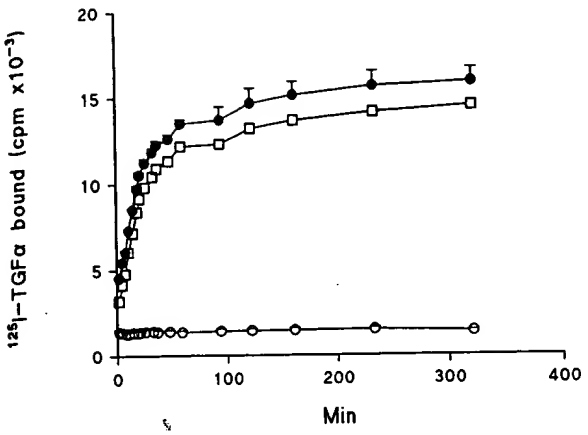

FIG. 4. Association of [^{125}I]TGFα to the membrane/bead complex. Membrane/bead complex and [^{125}I]TGFα (0.64 μCi/ml, 0.167 nM) were incubated in the absence (●) or presence (○) of 82 nM unlabeled EGF and at various time intervals specific binding (defined as binding in the presence of 82 nM EGF subtracted from binding in the absence of 82 nM EGF) was determined (□). Values represent means ± SE (n = 3).

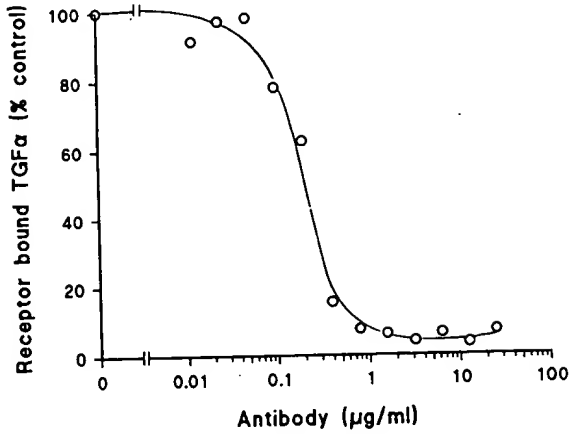

FIG. 6. Inhibition of [^{125}I]TGFα binding to the membrane/bead complex by anti-EGF receptor antibody clone 528. Membrane/bead complex and [^{125}I]TGFα were incubated with antibody and specific binding was determined. Values represent means of triplicate determinations (SE values <5%).

The data from the dissociation and association rate constants were then used to calculate K_d values. Since the data from the competition experiments were best expressed in terms of a monoexponential model, this model was initially used to determine the K_d value from the dissociation and association rate constants. Since $k_{obs} = (k_2 + k_1[D])$, where $[D]$ is the concentration of TGFα = 0.167×10^{-9} M, the rate constant of association, k_1, was calculated as 3.29×10^8 M^{-1} min^{-1}. $K_d = k_2/k_1$, and hence the K_d can be determined to be 0.11 nM. This value is similar to the K_d values determined from the competition curves. Performing a similar calculation using data from the biexponential fits gave K_d values of 0.021 and 0.50 nM.

We next examined the effects of antibodies on the binding of [^{125}I]TGFα. Monoclonal antibody clone 528 to the EGF receptor, which inhibits EGF binding (24), inhibited binding of [^{125}I]TGFα to SPA beads with an IC50 of 0.20 μg/ml (Fig. 6). Monoclonal antibody clones 29.1 and EGFR1 to the EGF receptor do not inhibit EGF binding (25,26). Neither clone 29.1 nor clone EGFR1 inhibited [^{125}I]TGFα binding at concentrations up to 25 μg/ml. Monoclonal antibody clone 134A-2B3 to TGFα did not reduce binding of [^{125}I]TGFα at concentrations up to 25 μg/ml.

During our evaluation of the TGFα SPA system we identified several agents that modulate TGFα binding. Suramin inhibited [^{125}I]TGFα binding (IC50 = 0.20 mM) (Fig. 7). The ether lipids, 1-*O*-hexadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine, 1-*O*-octadecyl-2-*O*-methyl-

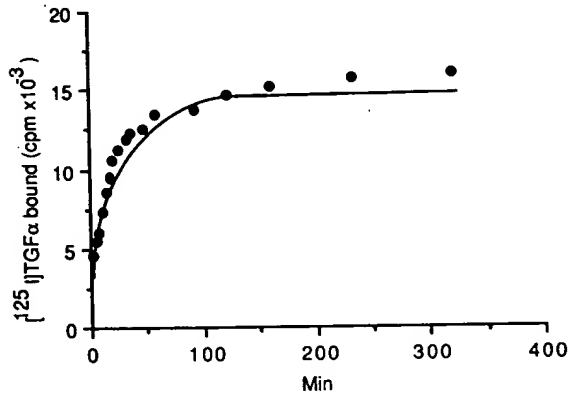

FIG. 5. Association of [^{125}I]TGFα to the membrane/bead complex drawn from the curve generated by KINETIC assuming a biexponential model.

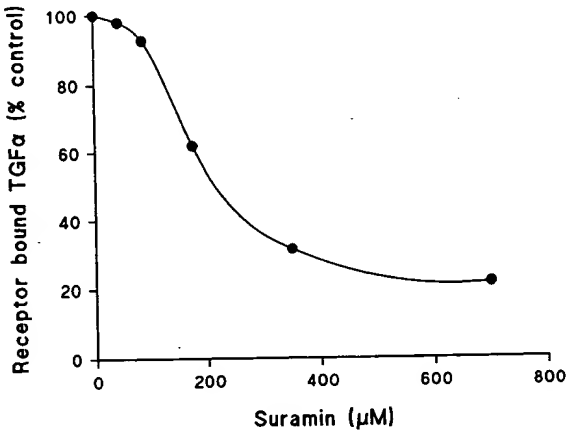

FIG. 7. Inhibition of [^{125}I]TGFα binding to the membrane/bead complex by suramin. Membrane/bead complex and [^{125}I]TGFα were incubated with suramin and specific binding was determined. Values represent means of triplicate determinations (SE values <5%).

sn-glycero-3-phosphocholine, and *rac*-lyso-platelet activating factor, reduced [^{125}I]TGFα binding with IC50 values of 49, 69, and 57 μM, respectively (means of two experimental determinations).

DISCUSSION

The binding of [^{125}I]TGFα to the SPA beads is characteristic of a receptor interaction. The A431 membranes bound on the SPA beads have a high affinity receptor site for TGFα with an equilibrium dissociation constant of 0.10–0.26 nM based on analysis of the competition data and association and dissociation rate constants. A second class of receptor was detected in association and dissociation experiments, but data obtained from competition experiments suggest that the second site probably represents a binding artifact.

In contrast to the large number of reports on EGF binding to its receptor, there are relatively few reports on the binding characteristics of TGFα. This is surprising in view of the greater importance of TGFα in tumorigenesis. Early work by Massague (27) identified two classes of TGFα binding sites on A431 membranes with K_d values of 0.025–0.03 nM and 0.18 nM. By contrast, Marquardt *et al.* (28) identified a single class of receptor on formalin-fixed A431 cells with a K_d of 2.7 nM. Results from Winkler *et al.* (29) indicated that TGFα had about half the receptor binding activity of EGF. In the present study, ligand binding characteristic have been obtained from lyophilized membrane preparations and further work is necessary to determine if lyophilization alters the binding characteristics.

Our studies with anti-EGF receptor antibodies are comparable to previous reports on the abilities of the antibodies to block [^{125}I]EGF binding. Our data therefore suggest that the anti-EGF receptor antibodies clones 29.1, 528, and EGFR1 do not distinguish between EGF and [^{125}I]TGFα binding. Evidence that an antibody can distinguish between binding of EGF and TGFα to purified human EGF receptors has recently been presented (21). The inability of the anti-TGFα antibody to block [^{125}I]TGFα binding was less expected since an anti-TGFα antibody blocked the binding of TGFα to A431 cells (10). One possible explanation is that the anti-TGFα antibody used in our experiments binds at a site other than the ligand binding domain.

We have used SPA to evaluate whether the antitumor activity associated with some ether lipids (30) could be mediated by modulation of TGFα binding. Ether lipids have multiple effects on cells, including inhibition of growth factor-dependent inositol phosphate signalling (31) and inhibition of estradiol uptake and TGFα secretion in the breast cancer cell line MCF7 (32). There was no significant difference in the inhibition of TGFα binding between the cytotoxic analogue 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine and the noncytotoxic analogue *rac*-lyso-platelet activating factor in our experiments. Thus inhibition of TGFα binding does not seem to be able to account for the cytotoxic action of 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine.

Suramin has anti-proliferative activity and, like the ether lipids, it has multiple cellular effects, including the blocking of growth factor–receptor interactions and inhibition of enzymes (33). The IC50 value for inhibition of TGFα binding by suramin determined in the SPA is clinically achievable, indicating that inhibition of TGFα binding is a potential site of action of this compound. The IC50 value for inhibition of TGFα binding is approximately three times lower than the concentration of suramin reported to block binding of [^{125}I]EGF to receptors on AKR 2B cells (17).

Although SPA can be used to obtain biological information on existing substances, it is particularly useful in screening for novel inhibitors of TGFα. In conjunction with automated liquid handling devices, we have routinely used SPA to screen up to a thousand samples a day. The assay eliminates many of the time-consuming steps associated with traditional radioligand binding assays such as tissue culture and membrane preparation and washing steps to separate bound from free ligand. However, further assays are required to determine whether the TGFα inhibitors are acting as agonists or antagonists. In conclusion, SPA is a convenient method for detecting inhibitors of TGFα binding to its receptor.

ACKNOWLEDGMENTS

We thank Gerry O'Bierne for helpful discussions and Nigel Bosworth for providing information on the SPA beads. We gratefully acknowledge Amersham International plc for the supply of SPA beads/receptor complex and methodology on their use.

REFERENCES

1. Neal, D. E., Bennett, M. K., Hall, R. R., Marsh, C., Abel, P. D., Sainsbury, J. R. C., and Harris, A. L. (1985) *Lancet* **1**(8425), 366–368.
2. Ozanne, B., Richards, S., Hendler, F., Burns, D., and Gusterson, B. (1986) *J. Pathol.* **149**, 9–14.
3. Smith, K., Fennelly, J. A., Neal, D. E., Hall, R. R., and Harris, A. L. (1989) *Cancer Res.* **49**, 5810–5815.
4. Nicholson, S., Sainsbury, J. R. C., Halcrow, P., Chambers, P., Farndon, J. R., and Harris, A. L. (1989) *Lancet* **1**(8631), 182–184.
5. Carpenter, G., and Wahl, M. I. (1990) *in* Peptide Growth Factors and Their Receptors (Sporn, M. B., and Roberts, A. B., Eds.), Vol. I, pp. 69–171, Springer-Verlag, Berlin.
6. Arteaga, C. L., Hanauske, A. R., Clark, G. M., Osborne, C. K., Hazarika, P., Pardue, R. L., Tio, F., and Von-Hoff, D. D. (1988) *Cancer Res.* **48**, 5023–5028.
7. Derynck, R., Goeddel, D. V., Ullrich, A., Gutterman, J. U., Williams, R. D., Brinman, T. S., and Berger, W. H. (1987) *Cancer Res.* **47**, 707–712.
8. Mydlo, J. H., Michaeli, J., Cordon-Cardo, C., Goldenberg, A. S., Heston, W. D. W., and Fair, W. R. (1989) *Cancer Res.* **49**, 3407–3411.

9. Petrides, P. E., Bock, S., Bovens, J., Hofmann, R., and Jaske, G. (1990) *Cancer Res.* **50,** 3934–3939.
10. Imanishi, K., Yamaguchi, K., Kuranami, M., Kyo, E., Hozumi, T., and Abe, K. (1989) *J. Natl. Cancer Inst.* **221,** 220–223.
11. Yoshida, K., Kyo, E., Tsuda, T., Tsujino, T., Ito, M., Niimoto, M., and Tahara, E. (1990) *Int. J. Cancer* **45,** 131–135.
12. Jhappan, C., Stahle, C., Harkins, R. N., Fausto, N., Smith, G. H., and Merlino, G. T. (1990) *Cell* **61,** 1137–1148.
13. Matsui, Y., Halter, S. A., Holt, J. T., Hogan, B. L. M., and Coffey, R. J. (1990) *Cell* **61,** 1147–1155.
14. Sandgren, E. P., Luetteke, N. C., Palmiter, R. D., Brinster, R. L., and Lee, D. C. (1990) *Cell* **61,** 1121–1135.
15. Darlak, K., Franklin, G., Woost, P., Sonnenfeld, E., Twardzik, D., Spatola, A., and Schultz, G. (1988) *J. Cell. Biochem.* **36,** 341–352.
16. Eppstein, D. A., Marsh, V. M., Schryver, B. B., and Bertics, P. J. (1989) *J. Cell. Physiol.* **141,** 420–430.
17. Coffey, R. J., Leof, E. B., Shipley, G. D., and Moses, H. L. (1987) *J. Cell. Physiol.* **132,** 143–148.
18. Carpenter, G. (1987) *Annu. Rev. Biochem.* **56,** 881–914.
19. King, I., Cornell, L., and Catino, J. J. (1990) *Proc. Am. Assoc. Cancer Res.* **31,** Abstract 2568.
20. Bosworth, N., and Towers, P. (1989) *Nature* **341,** 167–168.
21. Winkler, M. E., O'Connor, L., Winger, M., and Fendly, B. (1989) *Biochemistry* **28,** 6373–6378.
22. Munson, P. J., and Rodbard, D. (1980) *Anal. Biochem.* **107,** 220–239.
23. McPherson, G. A. (1985) *J. Pharmacol. Methods* **14,** 213–228.
24. Kawamoto, T., Sato, J. D., Le, A., Polikoff, J., Sato, G. H., and Mendelsohn, J. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 1337–1341.
25. Schlessinger, J., Lax, I., Yarden, Y., Kanety, H., and Libermann, T. A. (1984) *in* Receptors and Recognition (Greaves, M. F., Ed.), Vol. 17, pp. 279–303, Chapman & Hall, London.
26. Waterfield, M. D., Mayes, E. L. V., Stroobant, P., Bennet, P. L. P., Young, S., Goodfellow, P. N., Banting, G. S., and Ozanne, B. (1982) *J. Cell. Biochem.* **20,** 115–161.
27. Massague, J. (1983) *J. Biol. Chem.* **258,** 13,614–13,620.
28. Marquardt, H., Hunkapiller, M. W., Hood, L. E., and Todaro, G. J. (1984) *Science* **223,** 1079–1082.
29. Winkler, M. E., Bringman, T., and Marks, B. J. (1986) *J. Biol. Chem.* **261,** 13,838–13,843.
30. Berdel, W. E., and Munder, P. G. (1987) *in* Platelet-Activating Factor and Related Lipid Mediators (Snyder, F., Ed.), pp. 449–467, Plenum, New York/London.
31. Seewald, M. J., Olsen, R. A., Sehgal, I., Melder, D. C., Modest, E. J., and Powis, G. (1990) *Cancer Res.* **50,** 4458–4463.
32. Kosano, H., Yasutomo, Y., Kugai, N., Nagata, N., Inagaki, H., Tanaka, S., and Takatani, O. (1990) *Cancer Res.* **50,** 3172–3175.
33. La Rocca, R. V., Stein, C. A., and Myers, C. E. (1990) *Cancer Cells* **2,** 106–115.